



Histological evaluation of an alternative method of neophalloplasty based on two lower abdominal skin flaps and simultaneous buccal mucosa graft in the ventral surface of the neophallus (two-stage urethroplasty): Experimental study in rabbits

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Abstract *Objective:* To evaluate, in an experimental study in rabbits, a new model of neophalloplasty based on two lower abdominal skin flaps and ventral buccal mucosa graft for planned two-stage urethroplasty procedure.

Material and methods: Sixteen rabbits were operated and divided into four equal groups which were sacrificed at 2, 4, 8 and 12 weeks. The inflammatory pattern, presence of sub-epithelial fibrosis and epithelial changes in the grafted area were evaluated histologically.

Results: There were no deaths and no dehiscence of the wound was seen. One animal in the 2-week group developed an ulcer in the grafted area. We found minimal contracture of the neophallus, but this was not statistically significant between groups. Buccal mucosa graft showed good uptake in all groups, with vascular support from subcutaneous tissue of the flaps. The grafted area developed epithelial metaplasia, showing a decrease in cell layers with time, with disappearance of the sub-epithelial papillae and appearance of stratum granulosum and keratinization of the epithelial graft surface. A decrease in sub-epithelial fibrosis with replacement of immature by mature (eosinophilic) collagen was found. In the later groups was also observed an important decrease in inflammatory response, and the chorion of the grafted area presented a dilated capillary network, indicating that the process of neoangiogenesis was effective.

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Conclusion: Buccal mucosa displayed histological integration in the abdominal flaps with epithelial metaplasia in all groups. The surgical aspect of the neophallus was cosmetically acceptable, with minimal contracture.

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Introduction

Penile agenesis is a devastating condition and patients affected by this rare malformation are faced at birth with the same gender assignment dilemma as patients with ambiguous genitalia, both being considered as disorders of sexual differentiation [1–6]. Aphallia occurs at a rate of one case in every 30 million births [2,3] and consists of a flaw in the development of the genital tubercle, which is the precursor of the penis and clitoris [2].

Psychosexual development and gender identity seem to be directly linked to prenatal and early postnatal androgen exposure. Therefore, gender reassignment surgery frequently results in sexual dysphoria, and eventually may culminate with patient-initiated gender reassignment to male [3].

Phalloplasty is a formidable task in the treatment of perineal trauma, congenital defect and transsexual cases. Surgical techniques in adults have evolved from the classic tubed flaps to microsurgical transfers, creating not only a normal-shaped phallus but also a functional one, with neourethroplasty and the possibility of artificial erections

when prostheses are employed. In the pediatric population, however, mainly in patients with aphallia, the concept of early penile reconstruction and avoidance of feminization surgery is still developing. Recently, we published our preliminary experience with a technique based on a supra-pubic flap and tubularized buccal mucosa for the neourethra [3].

We investigated in this experimental model an alternative procedure based on two lower abdominal skin/subcutaneous tissue flaps and ventral buccal mucosa grafting for two-stage neourethroplasty. We did not perform the second step of the urethroplasty in this study, since the main objective at this time was to investigate whether it was possible to achieve histological integration of the buccal mucosa graft into the abdominal skin flaps and to evaluate the macroscopic aspect of the neophallus.

Material and methods

A total of 16 white New Zealand rabbits aged approximately 8 weeks and with a weight between 2.5 and 3.0 kg were

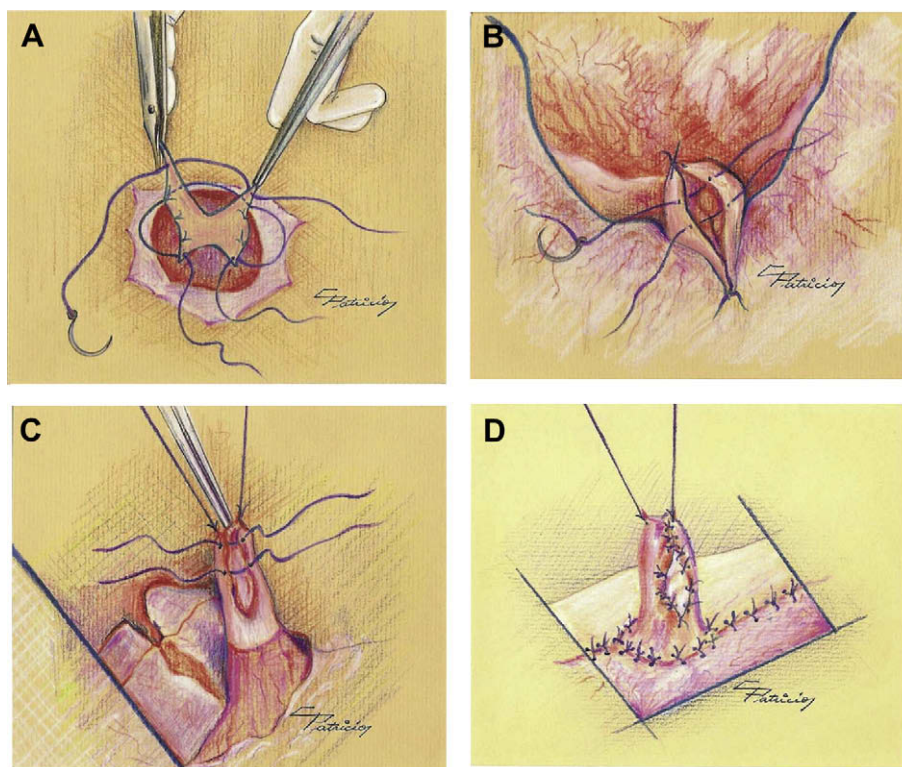


Figure 1 (A) The two abdominal flaps. (B) Suture of lateral edges of the flaps, defining the dorsal surface of the neophallus. (C) Suture of the medial edges of the flaps, defining the ventral surface of the neophallus, leaving a non-sutured area to graft fixation. (D) Final surgical aspect.

Table 1 Degree of inflammatory response in the grafted area.

Inflammatory score	Histological findings
0	Absence of inflammatory infiltrate
1+	Presence of one to four leukocytes/lymphocytes per field of high magnification
2+	Presence of more than four and less than 20 leukocytes/lymphocytes per field of high magnification
3+	Presence of more than 20 leukocytes/lymphocytes per field of high magnification
4+	Leukocytes/lymphocyte aggregate with formation of abscesses

divided into four groups and submitted to neophalloplasty. Each group consisted of four rabbits, which were sacrificed after 2, 4, 8 and 12 postoperative weeks when histological evaluation was performed.

The rabbits were anesthetized intramuscularly with ketamine hydrochloride (30 mg/kg) and xylazine (5 mg/kg). Subcutaneous enrofloxacin was administered at the beginning of surgery and daily for 7 days. After an adequate level of anesthesia was achieved and under sterile conditions, two 3.0 cm × 2.0 cm island flaps from the lower abdominal wall were defined and medially approximated (Fig. 1A). The lateral edges of each flap were sutured, defining the dorsal surface of the neophallus (Fig. 1B), whereas suturing the medial ones defined the ventral surface of the neophallus (Fig. 1C). A groove was left in the ventral surface of the neophallus, and a 1.0 × 0.5 cm free buccal mucosa graft

secured with interrupted 7.0 Monocryl sutures, leaving the subcutaneous tissue of the flaps as a bed for vascular integration of the graft (Fig. 1D). Abdominal wall was closed with interrupted 2.0 polyglycolic acid sutures. The graft was outlined from the inner cheek before infiltrating its margins with 1:100,000 epinephrine solution. The graft was harvested minimizing fat on its undersurface while avoiding dissection into muscle. The donor site was not sutured. A compressive dressing remained in place for 7 days. At the scheduled sampling time, the neophallus was measured and animals were sacrificed by injection of an overdose of ketamine.

The neophallus was removed and fixed in 10% formaldehyde. Transverse sections of 5.0 mm were obtained, processed into paraffin blocks, serially sectioned and stained with hematoxylin and eosin and Masson's trichrome, to localize collagen. An experienced pathologist (RD) examined the specimens and evaluated the inflammatory pattern, presence of sub-epithelial fibrosis, epithelial changes in the buccal mucosa graft and other findings. The pathologist was not informed which group the animal was in before the evaluation.

The inflammatory response was classified as acute, when there was infiltration of polymorphonuclear cells, or chronic, with infiltration by lymphomononuclear cells. A semi-quantitative assessment method was used, graduated from 0 to 4+: 0 = absence of inflammatory infiltrate; 1+ = minimal inflammation; 2+ = moderate inflammation; 3+ = marked inflammation; and 4+ = acute inflammation with leukocytes/lymphocyte aggregates forming micro-abscesses (Table 1). The foreign body reaction type was characterized by the presence of multinucleated giant cells. The degree of sub-epithelial fibrosis was analyzed also in a semi-quantitative way, graduated from 0 to

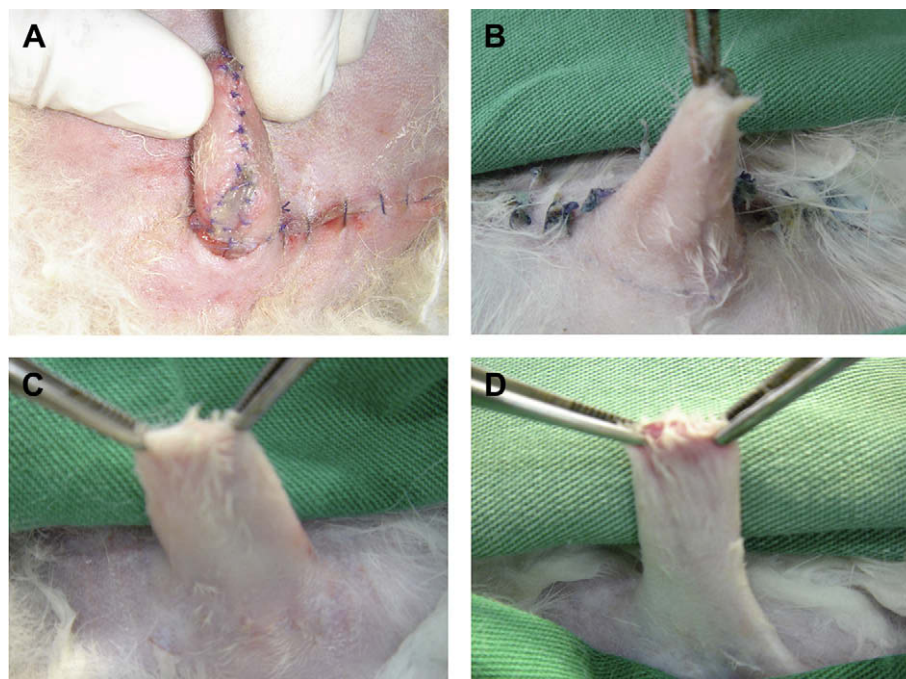
**Figure 2** Macroscopic aspect of the neophallus: (A) 2 weeks, (B) 4 weeks, (C) 8 weeks and (D) 12 weeks.

Table 2 Final length of neophallus.

	Group I	Group II	Group III	Group IV
	2 weeks	4 weeks	8 weeks	12 weeks
Animal 1	2.5 cm	2.5 cm	2.7 cm	2.6 cm
Animal 2	2.6 cm	2.9 cm	2.5 cm	2.5 cm
Animal 3	2.7 cm	2.5 cm	2.9 cm	2.8 cm
Animal 4	2.7 cm	2.9 cm	2.5 cm	2.9 cm
Mean	2.63 cm	2.70 cm	2.65 cm	2.70 cm

3+: 0 = no scarring fibrosis; 1+ = minimal scarring fibrosis; 2+ = moderate scarring fibrosis; and 3+ = pronounced scarring fibrosis. A quantitative analysis was performed to evaluate epithelial thickness and sub-epithelial fibrosis in the grafted area through the use of a microscopic ruler in one randomized animal of each group.

We performed statistical evaluation of the final length of the neophallus. The inferential analysis employed was analysis of variance, and statistical analysis was performed with the Statistical Package for Social Sciences version 11.0 for Windows.

Results

Macroscopic evaluation

No infection or dehiscence of the wound was seen. None of the animals presented dorsal contracture of the neophallus. One rabbit in the first group of sacrifice (2 weeks) developed an ulcer in the grafted area. The macroscopic aspect of the neophallus through the follow-up period is illustrated in Fig. 2.

Inferential analysis showed that the final length of the neophallus (cm) was statistically similar between groups

($P = 0.914$), showing minimal contracture (size reduction) (Table 2).

Microscopic evaluation

Buccal mucosa graft showed uptake within all groups, getting vascular support from the subcutaneous tissue of the flaps. The grafted area developed epithelial metaplasia, as an adaptive response to the new environment. This process determined the appearance of the granulosum layer and keratohyalin granules which were responsible for the keratinization process. This layer was not seen in buccal mucosa before grafting. The epithelium became thinner, with disappearance of the epidermal crests, still without skin appendages. Mean epithelial thickness reduced from 0.25, 0.1, 0.05 and 0.03 mm in the 2-, 4-, 8- and 12-week group, respectively (Fig. 3).

A decrease in the inflammatory infiltrate over time was found, a minimal to marked infiltrate in the 2-week group and an absence of inflammatory infiltrate in the 8- and 12-week groups being observed. This pattern appeared at the expense of lymphomononuclear cell infiltration (Fig. 4).

A decrease in collagen deposit was also verified. In the 2-week group, we observed moderate (2+) to marked (3+) sub-epithelial fibrosis and in the later groups (8 and 12 weeks) minimal fibrosis. The collagen deposit thickness was 3.7, 0.8, 0.4 and 0.6 mm in the groups of 2, 4, 8 and 12 weeks, respectively (Fig. 5). The collagen that was initially immature (fibrillar), rich in cells (young fibroblasts) and less eosinophilic, as observed in the groups of 2 and 4 weeks, was replaced by mature collagen, eosinophilic and filled by mature fibroblasts, in the later groups (Fig. 6).

In the 12-week group clear signs of neoangiogenesis were observed: a sub-epithelial dilated capillary network, without any sign of thrombosis of microvasculature or perivascular lymphocytic infiltration (Fig. 7).

The histological results are summarized in Table 3.

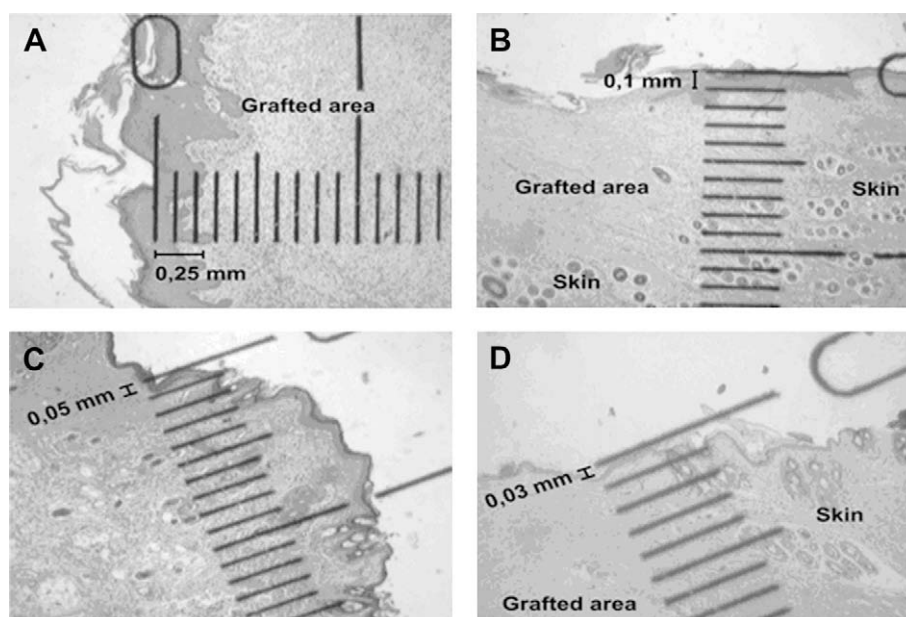


Figure 3 Hematoxylin and eosin $\times 25$, epithelial thickness: (A) 2 weeks, (B) 4 weeks, (C) 8 weeks and (D) 12 weeks.

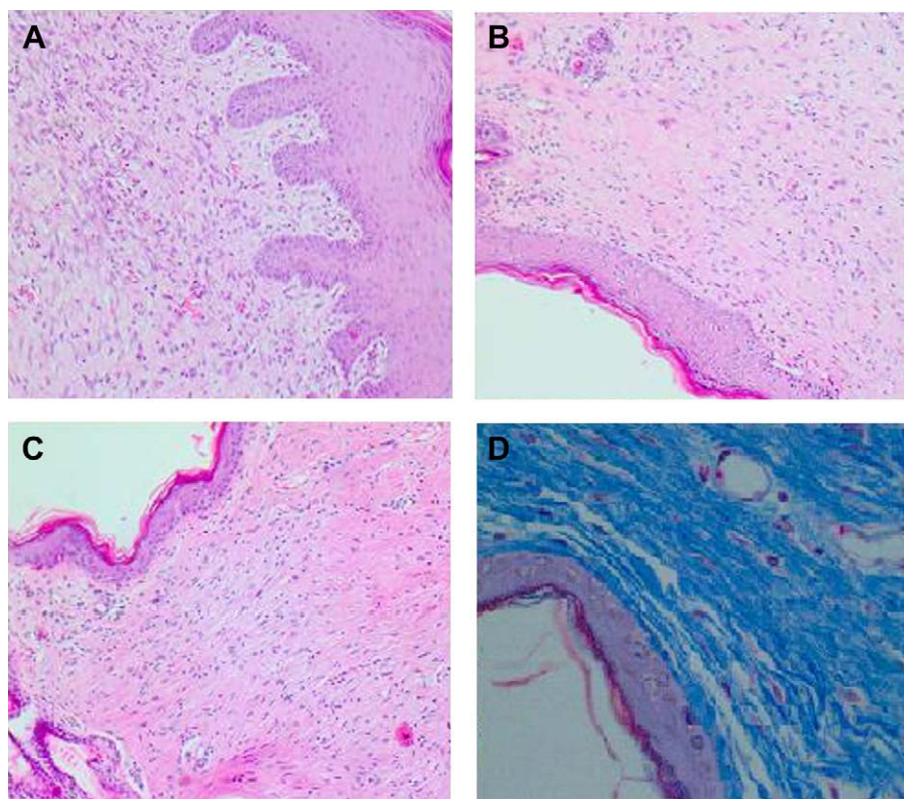


Figure 4 Hematoxylin and eosin $\times 100$, inflammatory infiltrate in grafted area: (A) 2 weeks, (B) 4 weeks, (C) 8 weeks and (D) 12 weeks.

Discussion

In the past, infants with congenital aphallia and those affected by cloacal exstrophy underwent early gender reassignment surgery, including bilateral orchiectomy,

urethral transposition, vaginal replacement and labial construction. Patients who have undergone total penectomy due to penile carcinoma or traumatic lesions have presented with severe psychological disorders [3]. Experimental data and several clinical observations have shown that prenatal

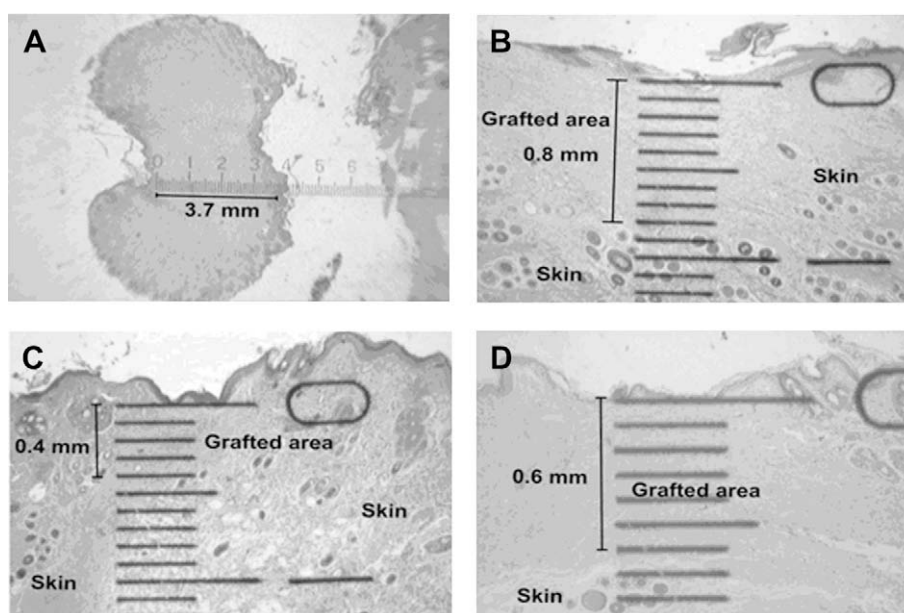


Figure 5 Hematoxylin and eosin $\times 25$, collagen deposit thickness: (A) 2 weeks, (B) 4 weeks, (C) 8 weeks and (D) 12 weeks.

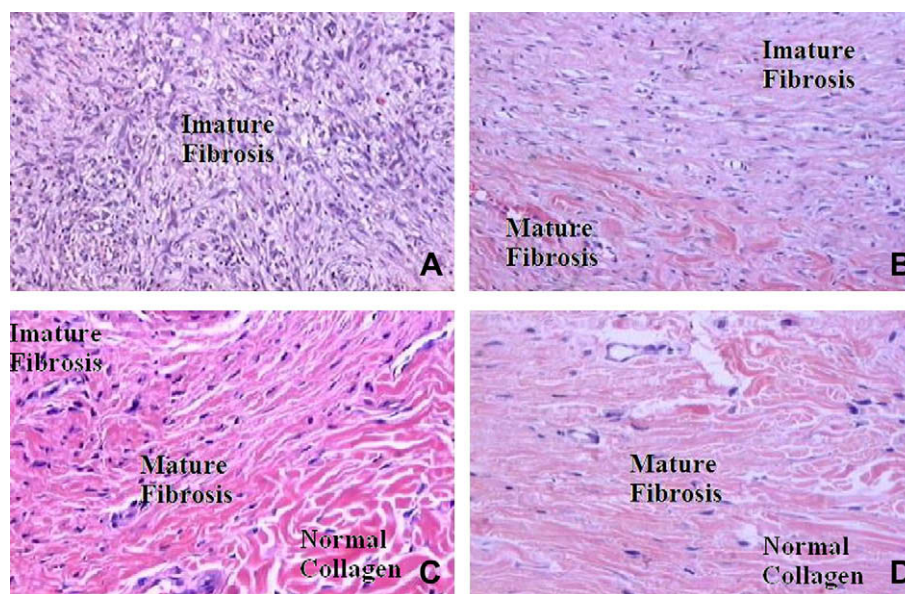


Figure 6 Hematoxylin and eosin $\times 100$, characteristics of fibrosis: (A) 2 weeks, (B) 4 weeks, (C) 8 weeks and (D) 12 weeks.

and early postnatal androgen exposure is crucial in psychosexual development and identity, and cannot be changed by neonatal castration, hormonal treatment, phenotypic adjustments or psychosexual manipulations.

The techniques of reconstructing the penis, urethra and glans in adults have gradually evolved from the classic tubed flaps to microsurgical transfers with primary and definitive reconstruction of the urethra inside the neopenis, and primary reconstruction of the glans and possibly the scrotum [7–9]. Microsurgical penile reconstruction is the gold standard surgical technique, frequently using the forearm fasciocutaneous flap. The main disadvantages of this technique are a large and unpleasant donor site scar, and the fact that this procedure can only be performed during post-pubertal period and in highly qualified centers [7–9].

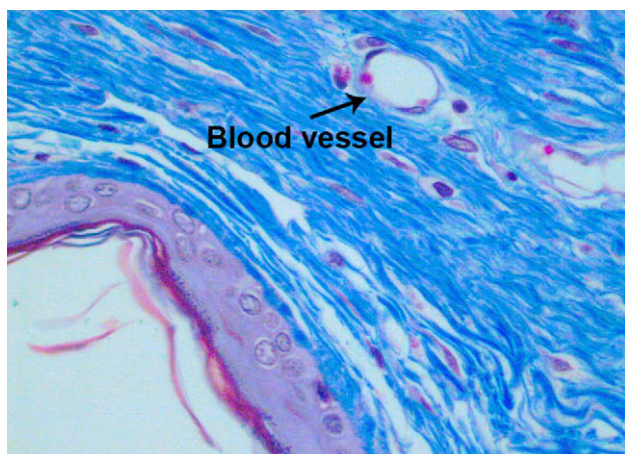


Figure 7 Masson's trichrome $\times 100$, dilated capillary network in the sub-epithelial portion of the grafted area, without vascular thrombosis or perivascular inflammatory infiltrate.

The multiplicity of techniques available for penile reconstruction makes it difficult to choose the right one for a particular patient. Provided that certain priorities are met concerning the quality of the neopenis, this choice probably depends on donor site morbidity, especially in children [10]. The use of a groin or lower abdominal flap to perform neophalloplasty has a clear advantage over other donor areas in limiting morbidity, as all of the resulting scar can be covered by underwear.

de Castro et al. [3] proposed neophalloplasty and neourethroplasty in children with penile agenesis using a quadrangular abdominal skin flap and tubularized buccal mucosa graft inside the tube formed by abdominal flap tubularization. This procedure can be performed even in small children, in contrast to microsurgical techniques that are commonly only used after 15 years of age, and does not necessitate a plastic surgeon with microsurgical expertise. Two Brazilian patients underwent the de Castro technique in our service. Both of them developed late urethral stenosis and dorsal contracture of the neophallus. These findings stimulated us to think of a different approach but using the same guiding principles.

Buccal mucosa has emerged as the tissue of choice for urethral reconstruction when local genital skin is unavailable. It has gained acceptance for its unique histological features, particularly its thick, non-keratinized epithelial layer and thin, highly vascularized lamina propria, which facilitates graft imbibition, inosculation and angiogenesis. The epithelium is non-keratinized, without a granulosum layer and keratohyalin granules. Histological and immunohistochemical studies have shown pronounced similarities between normal urethral and buccal mucosa with regard to comparative cytokeratin expression and immunoglobulin concentrations [11,12].

Graft take is theoretically more reliable in the staged approach rather than when buccal mucosa is used as onlay or tube. Neither onlays nor the ventral aspects of tubed grafts can be fixed to surrounding tissues and so they may

Table 3 Histological findings.

	2 weeks	4 weeks	8 weeks	12 weeks
Inflammatory infiltrate	1–3+	1+	0	0
Kind of inflammation	Chronic inflammation with infiltration by mononuclear inflammatory cells	Chronic inflammation with infiltration by mononuclear inflammatory cells		
Sub-epithelial fibrosis	2–3+	1–2+, with young fibroblasts	1+	1+
Bladder mucosa changes	Same number of cell layers with initial keratinization	Decrease in the number of cell layers with complete keratinization	Buccal mucosa similar to the adjacent skin with same number of cell layers and keratinized	Keratinized epithelium identical to adjacent skin
Fibrosis of subcutaneous fat	1+	1+	2+	3+
Other findings	Ulcerative formation in one rabbit	Edema with sub-epithelial hemorrhage and focal sebaceous gland metaplasia	Sebaceous gland metaplasia	

be more susceptible to adverse consequences from unwanted fluid collections. In two-staged procedures, following revascularization, the buccal tissue became a supple epithelial strip analogous to the urethral plate, creating a well vascularized substitute urethral plate for tubularization, with low complication rates and good cosmetic outcomes [12].

We performed a new phalloplasty technique, similar to de Castro [3], using two lower abdominal flaps to build the neophallus, and trying to decrease dorsal contracture. We believed that the addition of a new suture line would diminish the probability of dorsal contracture, in order to achieve entire tubularization of a unique abdominal flap. In our model, the buccal mucosa was attached to the ventral aspect of the neophallus, to compose the neourethral plate that would be tubularized in a second stage.

Histological evaluation confirmed integration between the buccal mucosa and the abdominal flaps. In the grafted area epithelial metaplasia [13] was verified, as an adaptive response to the new environment. A reduction in cell layers and keratinization were observed, the identification of a granulosum layer with keratohyalin granules being possible at the level of the epithelial line. The epithelial crests disappeared in the later groups and a huge capillary net was identified without any perivascular lymphocytic infiltration or microvascular thrombosis, revealing an effective process of neoangiogenesis. An ulcer was detected in only one rabbit showing poor vascularization of the grafted area from the subcutaneous tissue of the flaps.

The cicatricial collagen deposit and inflammatory infiltrate were also reduced in the later groups. The collagen that initially was immature (rich in young fibroblasts and less eosinophilic) was replaced by mature collagen. A different pattern was observed in the central axis of the neophallus, an increase in fibrous tissue being verified. This was shown macroscopically by a more rigid neophallus in the groups of 8 and 12 weeks.

The first priority in neophalloplasty is to construct a penis which will retain its shape and stiffness in the long term, and a functional structure with possibility to void. When the soft tissue used to construct the neophallus is not supported by a rigid structure, the penis ultimately contracts and loses its shape, and later implantation of a stiffener is very difficult or impossible.

Alloplastic material such as silicone rubber usually erodes through the penile skin and may be extruded. Some authors use an autologous vascularized bone segment to decrease subsequent resorption and guarantee stiffness to the neophallus, avoiding contracture [10]. We did not use a stiffener, and dorsal contracture was not verified in any animal operated. A longer follow up is necessary to be certain that a new suture line in the dorsal surface of the neophallus does reduce the dorsal contracture rate.

This study consisted of an experimental surgical model but the second stage of the neourethroplasty was not performed here. We believe that this procedure may be feasible based on the histological results of the research work.

Conclusion

Buccal mucosa free graft integrates when attached to the subcutaneous portion of the abdominal flap, developing epithelial metaplasia to become more resistant to the new environment. We believe that this well vascularized urethral plate may be tubularized in a second stage, as proposed by Bracka in the surgical repair of complex hypospadias, reducing the possibility of graft contracture and posterior urethral stenosis. The experimental feasibility of a new technique is not always reproducible in humans, especially in regard to clinical complications. We acknowledge this limitation of our method, but feel more confident now to develop this technique for surgical use, and hope to start clinical series in the near future.

Conflict of interest

The study was developed in cooperation between the disciplines of Urology, Technical Operative and Experimental Surgery and Department of Pathology at the Federal University of São Paulo. The surgical procedures were performed in the laboratory of Technical Operative and Experimental Surgery Department, and processing and histological analysis performed in pathology laboratory for research in the Department of Pathology.

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